THE LUMINESCENCE OF THE MILLIPEDE, LUMINODESMUS SEQUOIAE [1]

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The millipede *Luminodesmus sequoiae* was first described by Loomis and Davenport (1951). Following this, Davenport, Wootton and Cushing (1952) described the biology of the animal and the general nature of its luminescence. They found that light emission originates from cells in the deeper layers of the integument. The present paper describes a more detailed study of its luminescence.

The bioluminescent reaction in four different organisms (*Cypridina,* fireflies, bacteria and *Gonyaulax*) has been studied in recent years (Tsuji, Chase and Harvey, 1955; McElroy and Hastings, 1955; McElroy and Green, 1956; Hastings and McElroy, 1955; Strehler, 1955; Hastings and Sweeney, 1957). The common feature is that the reaction involves an enzymatic oxidation with molecular oxygen. Although it was demonstrated by McElroy that adenosine triphosphate is an absolute requirement for firefly luminescence, its possible role as an energy source in the reaction has not been clarified. In bioluminescent reactions in general it is assumed that the energy must be derived from the oxidation of a substrate, which is usually termed luciferin (*e.g., Cypridina* luciferin, firefly luciferin, etc.). None of the products of luminescent reactions have been definitely identified and the reactants as well as the enzymes are different in all cases studied. Such studies are of interest for the general problem of how the living cell transforms chemical energy into other forms of energy.

Materials and Methods

About 1000 animals were collected by a party of five on the nights of May 10 and 11, 1956, in the vicinity of Camp Nelson, Tulare County, California. The animals were abundant and readily visible to the dark-adapted eye by their own light on the surface of the ground in the forest. The animals were brought back to the laboratory and stored in glass containers with ample humus. Light intensity was measured with apparatus previously described (Hastings, McElroy and Coulombre, 1953), using a photomultiplier tube and automatic graphic recording.

Results

1. In vivo luminescence

Luminescence in *Luminodesmus* is continuous but fluctuating. The light intensity (recorded from single animals over long periods up to 24 hours) fluctuates

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by 20 to 40 per cent (or occasionally more) around a relatively constant mean. Since these light intensity changes could be detected with the eye, we are certain that they do not result from movements of the animal. A two-hour portion from such a recording is reproduced in Figure 1A. It can be seen that there is an instance when the light intensity doubled, apparently spontaneously. Such a marked increase in light intensity also occurs when the animal is handled. Indeed we found that striking the test tube in which the animal was placed would evoke such a response (Fig. 1B). The response still occurred immediately after the animal had been decapitated. Upon stimulation of the nerve cord which induced electrical shocks no luminescent response was observed which could be attributed to the effect of the electrical stimuli. The way in which the luminescent changes are brought about in the living animal is not clear.

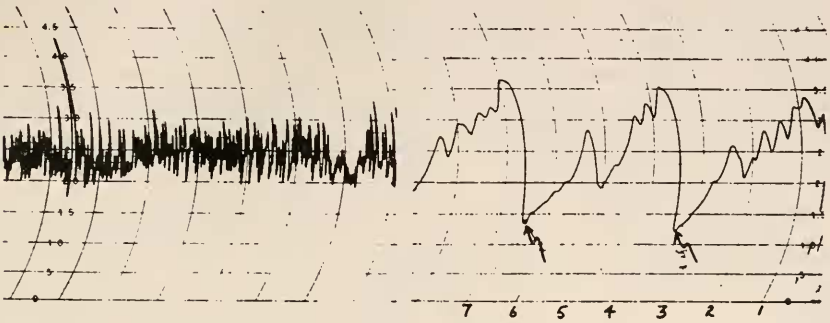

FIGURE 1. Reproductions of recordings of *Luminodesmus* luminescence. Ordinate, light intensity; abscissa, time, to be read from right to left in both cases. *Left:* Luminescence of an undisturbed animal over a two-hour interval. Time between vertical divisions, 15 minutes. *Right:* Luminescence changes of an animal where the test tube containing the animal was tapped lightly at the two instances noted by arrows. Time between vertical divisions, one minute.

Isolated pieces of the animal retain their luminescence for a long time. The intensity decreases gradually to about one half of its original value in 8 hours. Although fluctuations in intensity may occur for the first 15 minutes, it is essentially a steady luminescence thereafter. An eviscerated specimen from which the first few and last few segments are cut off behaves in essentially the same manner. Animals removed from humus and kept in a test tube for a day or more also showed little fluctuation in intensity. Whether or not this was due to water depletion or to starvation was not determined. Preparations with little or no light intensity fluctuation were used in the various experiments described below.

2. Possibility of luminous symbiotic bacteria

In some organisms luminescence arises from an association with luminous bacteria (see Harvey, 1952). The possibility that this might be the case in *Luminodesmus* was investigated some years ago by W. D. McElroy and one of us (J. W. H.). Whole animals and extracts of animals were put on agar plates containing a variety of media. Plates with a range of salt concentrations (0, 1%, 2% and 3% sodium chloride) were made up with both glycerol and glucose as carbon sources and Bacto Tryptone. No growth of luminescent bacteria occurred on any plate.

It may also be noted that the effect of varying oxygen concentration upon the luminescence of *Luminodesmus* (see section 5) indicates that the light is not bacterial in nature. In bacteria (Hastings, 1952; Shapiro, 1934) decreasing the oxygen concentration has no effect upon luminescence unless the concentration is less than about 0.3%.

3. Color of the light

The light emission from *Luminodesmus* is weak, requiring dark-adaptation on the part of the observer to see it. The emission spectrum was determined by placing a single animal at the entrance slit of a Bausch and Lomb Grating Monochromator, and the phototube at the exit slit. A second phototube was placed by the entrance slit to monitor any changes in the intensity of the animal during the course of the experiment. The entrance and exit slits were both set at 1 mm., which gives a dispersion of 12 mμ with the grating used (15,000 lines per inch). The light intensity was measured at the various wave-length settings and then corrected for phototube sensitivity and for monochromator efficiency. The corrected values are plotted against wave-length in Figure 2, giving the emission spectrum with the maximum in the green at 495 mμ. This spectrum is similar to that of some of the

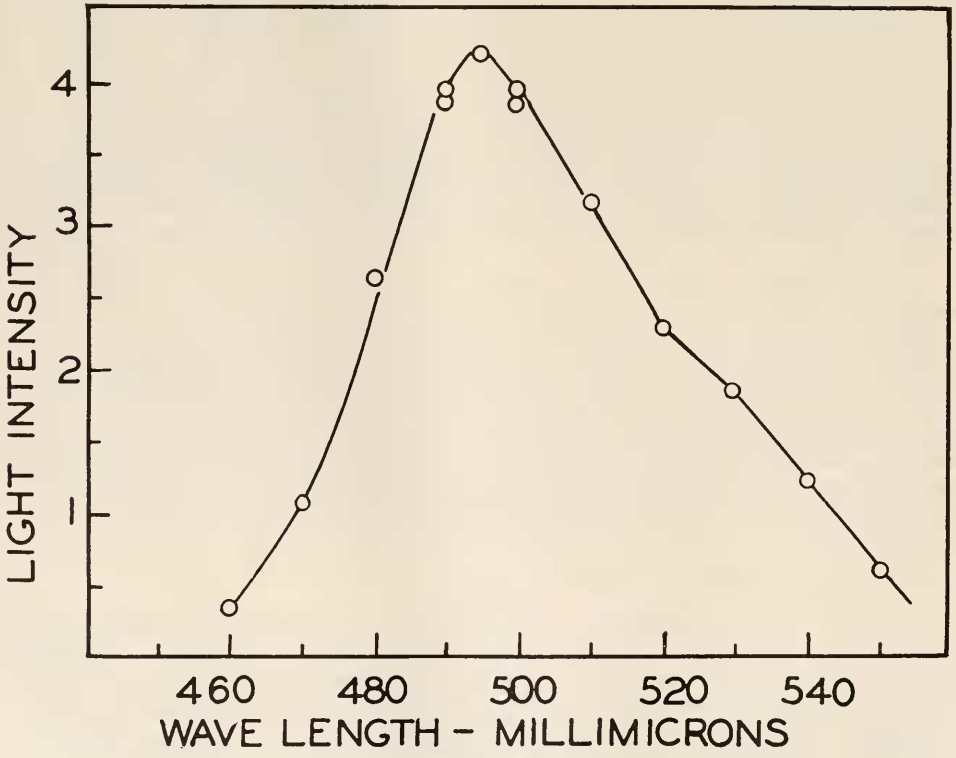

FIGURE 2. Emission spectrum of *Luminodesmus sequoiae*. Ordinate, light intensity in arbitrary units, corrected for phototube sensitivity and monochromator efficiency.

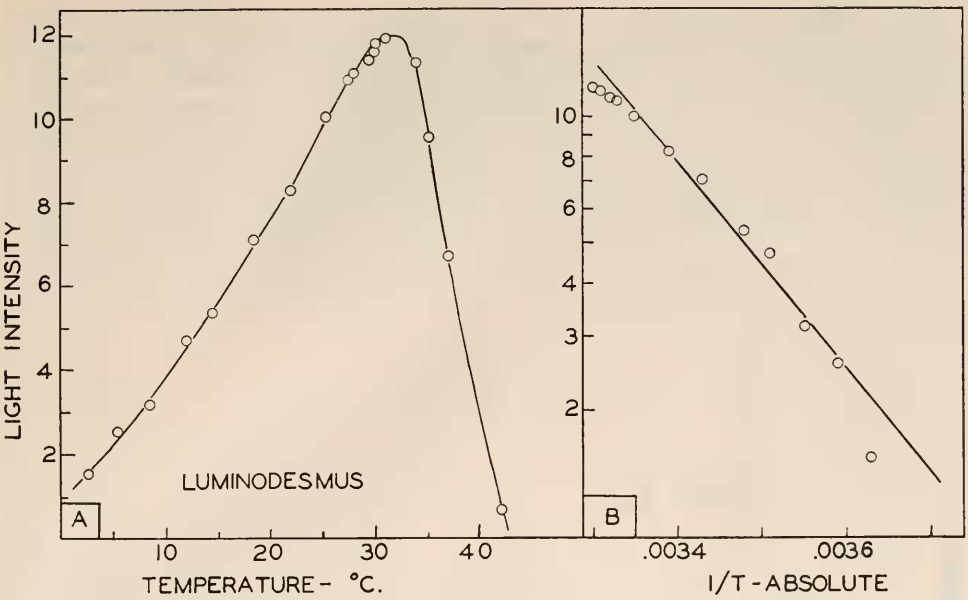

FIGURE 3. (A) Effect of temperature upon luminescence. Ordinate, light intensity in arbitrary units. See text for details. (B) Data of Figure 3A plotted according to the Arrhenius equation. Ordinate, light intensity in arbitrary units plotted on a log scale; abscissa, the reciprocal of absolute temperature.

luminous bacteria, although the spectrum of bacterial emission may depend upon the density of the suspension (Harvey, 1952).

4. Effect of temperature

The effect of temperature upon the luminescence was determined using both intact organisms and eviscerated specimens. The results were essentially the same in both cases. The specimen was held in place in a test tube by a cotton plug. Temperatures were adjusted by holding the tube in a water bath, and the temperatures plotted are those read from a thermometer placed in the tube beside the specimen. The tube was then quickly removed from the bath and placed in front of the phototube.

The data from one experiment with an eviscerated specimen are plotted in Figure 3, along with a plot of the data according to the Arrhenius equation. The Q_{10} for the process is 1.95 between 10° and 20°; 1.73 between 15° and 25°; and 1.55 between 20° and 30°. The sharp decrease in luminescence above the optimum of 31.5° is most likely the result of heat denaturation of enzymes. The activation energy for the over-all process may be calculated from the slope of the straight line drawn in the Arrhenius plot. The value obtained in this case is about 12,000 calories.

5. Effect of varying oxygen concentration

The effect of oxygen concentration upon the luminescence was determined quantitatively, using both whole animals and eviscerated specimens. Both gave

similar results. Luminescence was greatest in 100% oxygen and progressively decreased at lower concentrations, being reversibly extinguished in pure nitrogen, as reported by Davenport, Wootton and Cushing (1952). An oxygen concentration of about 6.5% decreased the intensity from that in air by about one half.

The specimen was held in place with cotton in a stoppered 10-ml. test tube, with glass tubing to bring the gas mixtures into the test tube. Gas mixtures of the desired oxygen concentration were prepared by mixing nitrogen with air or oxygen at measured rates, using calibrated flow-meters. The typical effect of lowered

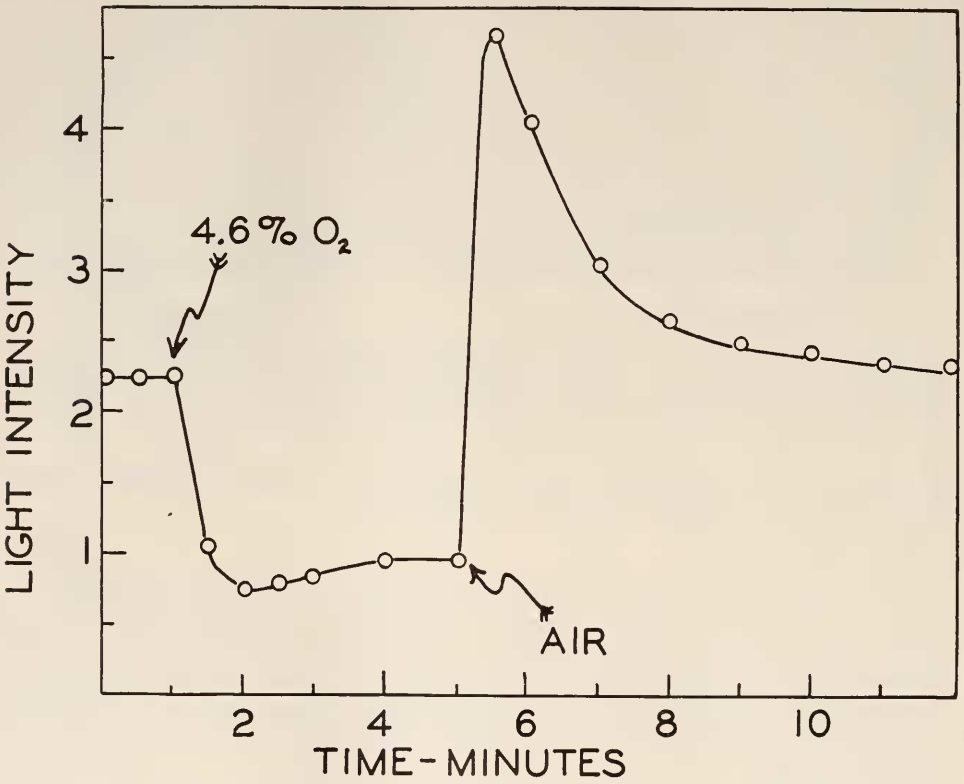

FIGURE 4. Changes in luminescence with time when a gas mixture containing 4.6% oxygen was passed over the animal for four minutes followed by the readmission of air.

oxygen concentration upon luminescence is shown in Figure 4. The response is essentially similar to that found in several other luminous organisms (Hastings, 1952; Hastings, McElroy and Coulombre, 1953; Hastings and Buck, 1956). There is a characteristic "undershooting" when the animal is exposed to a lowered oxygen concentration, and an overshoot or excess flash of luminescence, when it is exposed to a higher concentration. This excess luminescence is greater when a lower oxygen concentration is used during the period previous to the time when air is readmitted. For example, in the experiment shown in Figure 4, the luminescence was about twice the baseline level when air was admitted. With

1% oxygen the luminescence was 2.5 times and with 10% oxygen it was 1.5 times the baseline level. This suggests that the substrate for the luminescent reaction (luciferin) is the product of a series of relatively slow reactions. When the oxygen concentration is changed the luciferin comes to a new steady-state concentration, but only relatively slowly.

The values for light intensity versus oxygen concentration plotted in Figure 5 are the steady-state values, measured just previous to the time when the animal was returned to air. The data plotted are the results obtained with six different specimens. Although there was a variation in the results, the data for any given

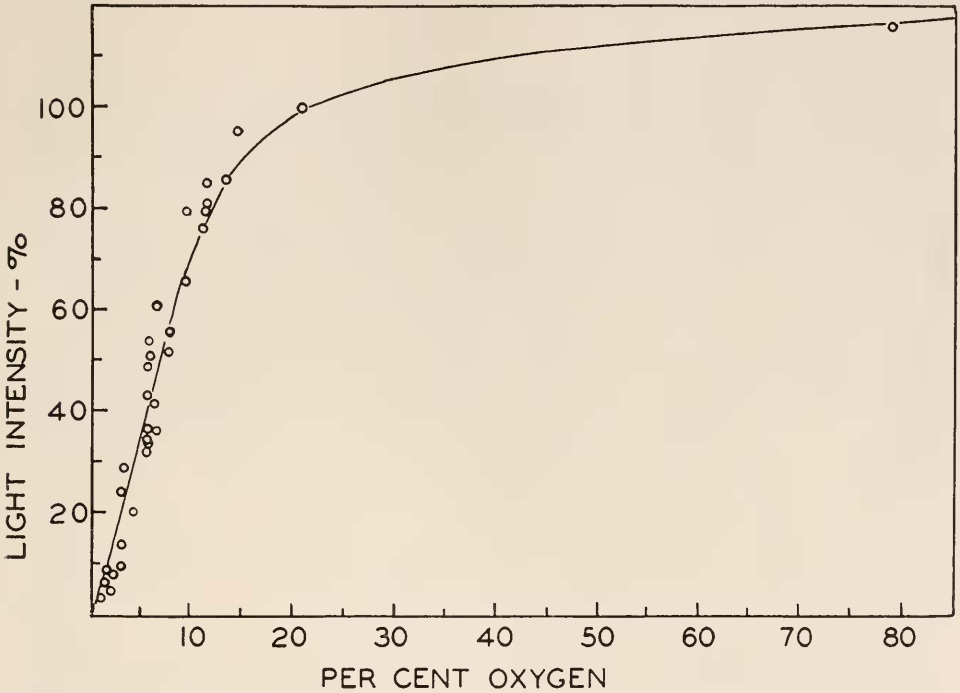

FIGURE 5. Effect of oxygen concentration upon the steady-state luminescence of *Luminodesmus*. Data are taken from experiments such as are illustrated in Figure 4. The luminescence, in low or high oxygen just prior to the readmission of air, is expressed as the per cent of the steady-state of luminescence in air.

animal fall along a smooth curve, suggesting a real difference between one animal and another, rather than error in the method. The reason for these differences could not be ascertained. Neither carbon monoxide nor carbon dioxide when added to the gas mixtures in 1% concentrations had any effect upon the shape of the curve for a given animal.

6. Fluorescence

Luminodesmus is highly fluorescent under ultraviolet light, and it was suggested previously (Davenport *et al.*, 1952) that this fluorescent compound might be in-

volved in the luminescent reaction. In luminous bacteria, for example, it has been demonstrated that reduced flavin mononucleotide ($FMNH_2$) is involved in the luminescent reaction, possibly as luciferin (McElroy, Hastings, Sonnenfeld and Coulombre, 1953; Strehler, Harvey, Chang and Cormier, 1954). FMN and other flavins are highly fluorescent in the oxidized form and are not at all fluorescent in the reduced form. Under anaerobic conditions in the intact animal the luciferin should be essentially 100% in the reduced state. However, there is absolutely no change in the fluorescence of *Luminodesmus* during anaerobiosis. The possibility that the fluorescence of the organism comes from a flavin compound involved in the luminescent reaction must therefore be ruled out.

7. Luminescence in extracts

Davenport, Wootton and Cushing (1952) reported negative results in all attempts to restore luminescence in filtered water extracts of *Luminodesmus*. Using sensitive light-measuring equipment, we have repeated their experiments and made additional studies with extracts. The most suitable method which we found for preparing extracts was to remove the gut from animals and dry them overnight in a vacuum desiccator with calcium chloride. The dry animals were then pulverized to a fine powder by grinding, and extracted with cold acetone. The acetone was removed by filtration and the dry powder kept in a vacuum desiccator. The powder remained active in this state for a period of at least two weeks.

When this powder was mixed with water a dim luminescence occurred (visible only to the dark-adapted eye), lasting for about 10 minutes, the half time for decay being about two minutes. Stirring always resulted in a temporary increase in luminescence, suggesting that leaching from particulate matter was taking place. Following stirring the light intensity returned to the original level. When such a solution was filtered through fine sintered glass (maximum pore size, 5.5 microns), the filtrate retained luminescence. Stirring did not affect the intensity of the filtrate, indicating that the particles from which leaching was occurring had been removed.

The intensity of luminescence in these extracts was dependent upon the pH of the solution, with an optimum at about pH 8.9. The determination was made by extracting equal quantities of the powder with 0.05 *M* trihydroxyaminomethane-maleic acid buffer at various pH values and measuring the light intensity of the solution. In all experiments described below a buffered extract at pH 8.9 was used, and buffered reagents where needed.

In the classical luciferin-luciferase test a fraction which has been extracted with hot water is combined with a cold water extract in which the luminescent reaction has been allowed to run to completion. In the hot water extract the enzyme has been destroyed, presumably leaving available substrate, or luciferin. In the cold water extract the luciferin has all been used up leaving active enzyme, or luciferase. The two mixed together should therefore give light, but with *Luminodesmus* completely negative results were obtained. Moreover, neither the hot water extracts nor exhausted cold water extracts, when added to a luminescing extract, had any effect upon the light intensity.

Attempts to separate the presumed luciferase and show its activity in the reaction were negative. Fractions were obtained by ammonium sulfate precipitation,

by alcohol fractionation, and by dialysis. None was active when added either to luminescing mixtures or to hot water extracts.

A large number of compounds were tested for their ability to modify the light intensity in luminescing extracts.[2] ATP was found to have appreciable activity. ATP added to an extract which was emitting light caused the intensity to increase by 10 to 30 per cent. The effect was not due to a pH change since buffered ATP solutions were used. It was effective with filtered extracts as well as unfiltered. Other pyrophosphate compounds were not tried, so it is possible that the action of ATP could be non-specific, similar to the effect of pyrophosphate compounds added secondarily to luminescing firefly extracts (McElroy, Hastings, Coulombre and Sonnenfeld, 1953). The fact that ATP would not restore luminescence to dark extracts indicates such a non-specific role. No restoration of luminescence occurred even when ATP was added to hot water extracts together with exhausted cold water extracts. However, when $MgSO_4$ was added following ATP addition, there was an additional increase in light intensity, suggesting the possibility of a more specific role for ATP. Also, 0.05 *M* Versene (ethylene diamine tetra acetic acid) was found to depress luminescence, indicating the possibility that the reaction is activated by a metal ion. All of the coenzymes listed in footnote 2 were tested in combination with ATP. None was found to have any stimulatory effect, although FMN and riboflavin slightly depressed luminescence.

The results give little clue as to the nature of the reaction. If a luciferin type compound is involved in a classical oxidative reaction, possibly in combination with ATP, then the luciferin must be highly unstable. In fact, such a highly unstable and heat-labile luciferin could account for the results we have obtained. The inhibition by flavins might mean that some flavin compound is involved in the reaction, although we would not expect a flavin to be particularly unstable.

Summary

1. The luminescence of *Luminodesmus* is continuous, but fluctuates by 20 to 40 per cent or more. The mechanism by which light emission is controlled is not known. No evidence was found for the suggestion that the light is bacterial in origin.

2. The luminescence is green with a maximum emission at 495 mμ and is optimal at a temperature of 31.5° C. Light emission is greatest in pure oxygen and extinguished in pure nitrogen. An oxygen concentration of 6.5% decreased the intensity from that in air by about one half.

3. Luminescence in water extracts of dried acetone powders has been demonstrated.

4. We have not been able to restore luminescence to dark extracts either by the classical luciferin-luciferase technique or by adding a variety of biochemical inter-

[2] Substances tested for activity, either singly or in combination, were: adenosine triphosphate (ATP), $MgSO_4$, riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide, oxidized and reduced diphosphopyridine nucleotide, oxidized and reduced triphosphopyridine nucleotide, coenzyme A, beef heart extract (Armour), yeast concentrate (Sigma), liver concentrate (Sigma), do-decyl aldehyde, ethyl alcohol, glycerol, glucose, glucose-1-phosphate, thiomalate, thiooctate, glutathione, cystine, cysteine, KCN, sodium arsenite, iodoacetate, sodium fluoride, sodium azide, p chloro-mercuro-benzoate, naphthoquinone, quinhydrone, hydroquinone, quinone, firefly extracts, NaCl, Na_2HPO_4, KH_2PO_4 and $MnSO_4$.

mediates. We have found that if adenosine triphosphate is added to extracts while they are luminescing an increase in light intensity occurs.

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